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[Original Article]

OVEREXPRESSION OF BOTH CLOCK AND BMAL1 INHIBITS ENTRY TO S PHASE IN HUMAN COLON CANCER CELLS

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Abstract : Many physiological, biochemical and behavioral processes operate under the circadian rhythm, which is generated by an internal time-keeping mechanism commonly referred to as the biological clock, in almost all organisms from bacteria to mammals. The core circadian oscillator is composed of an autoregulatory transcription-translation feedback loop, in which CLOCK and BMAL1 are positive regulators. A cell has two mechanisms, "cell cycle" and "cell rhythm", the relationship between which remains controversial. Therefore, the aim of this study was to explore the effect of *Clock* and *Bmal1* on cell cycle, especially on the G1 phase, using vectors with the tetracycline operator-repressor system. The present study revealed that simultaneous induction of *Bmal1* and *Clock* had an influential effect on the cell cycle in SW480/T-REx/*Clock*/*Bmal1* cells, in which both *Clock* and *Bmal1* could be induced by tetracycline. The observation that induction of both *Clock* and *Bmal1* inhibited cell growth and the significant increase of the G1 phase proportion of in SW480/T-REx/*Clock*/*Bmal1* cells indicated that entry from the G1 to S phase was inhibited by the induction of *Clock* and *Bmal1*. Furthermore, overexpression of *Clock* and *Bmal1* prevented the cells from entering into the G2/M phase induced by Paclitaxel, and made the cells more resistant to the agent. In conclusion, we found that overexpression of both *Clock* and *Bmal1* suppressed cell growth. In addition, the present study raised the possibility that *Clock* and *Bmal1* may in part play a role in preventing the cells from entering G1 to S phase of cell cycle via suppression of *CyclinD1* expression, and thus acquiring resistance to Paclitaxel.

Key words : circadian rhythm, *Clock*, *Bmal1*, cell cycle, paclitaxel

INTRODUCTION

Circadian regulation is a regulation under which many physiological, biochemical and behavioral processes operate. It is generated by an internal time-keeping mechanism commonly known as the biological clock, and exists in almost all organisms from bacteria to mammals[1,2]. Circadian rhythm is controlled by genetically determined networks of transcription-translation feedback loops involving sets of *Clock* genes[3,4], in which *Clock* and *Bmal1* function as positive regulators, and *Period (Per)* and *Cryptochrome (Cry)* act as negative ones[5-7]. The basic helix-loop-helix-PAS domain proteins CLOCK and BMAL1 form a heterodimer, and then activate transcription of *Per*, *Cry* and several other genes besides *clock* genes through their binding to an E-box (CACGTG) element at each promoter region[8,9]. In addition, these two molecules are indispensable for maintaining the circadian rhythm feedback loop[10,11]. After the PER and CRY proteins are translated in the cytoplasm, they form heterocomplexes and translocate into the nucleus and inhibit their own transcription[12-14].

In general, living cells are regulated by two different mechanisms, referred to as "circadian rhythm" and "cell cycle". Both are based on the conceptual device of interlocked autoregulatory loops. Furthermore, both are involved in therapy for patients with certain types of malignancy. "Cir-

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cadian rhythm" has recently been applied for chronotherapy which contributes to the reduction of side effects and enhancement of chemotherapy. Many studies have reported that the circadian clock could modulate the morbidity and the efficacy of anticancer therapy[15,16]. These findings led to the idea of chronotherapy as a way of optimizing the efficacy of anticancer drugs for cancer patients by the modification of drug administration at appropriate times of the day. Cell cycle is one of the main targets for anti-cancer drugs. "A large variety of drugs have been developed and analyzed in a cell cycle-dependent manner[17-19].

Although some reports have indicated the relationship between circadian rhythm and cell cycle at the G2/M checkpoint[20,21], the relationship between circadian rhythm and the G1 checkpoint is still unknown. The aim of this study was to explore the effect of the simultaneous induction of both *Bmal1* and *Clock* on the cell cycle, especially at the G1 checkpoint.

MATERIALS AND METHODS

Cells, Cell Culture and Chemicals

SW480 cells (Human colon cancer cell line) were provided by the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) 2 mM glutamine in a 5% CO_2 incubator at 37°C. Tetracycline (TET) was purchased from Invitrogen, and its stock solution was prepared in phosphate buffered saline (PBS) at a concentration of 10 mM. Dexamethasone was purchased from Sigma-Aldrich, and its stock solution was prepared in ethanol at a concentration of 2 mM. Blasticidine was purchased from Invitrogen, and its stock solutions were prepared in dimethylsulfoxide (DMSO) at a concentration of 10 mM. Paclitaxel (PTX) was purchased from Sigma, and its stock solution was prepared in DMSO at a concentration of 1 mM.

Cloning of cDNA of Clock and Bmal1, and Plasmid Construction

cDNA of human *Clock* and *Bmal1* was amplified by PCR using human placental cDNA. The amplified human *Clock* and *Bmal1* cDNA was then cloned into the EcoRI and NotI restriction sites of the pcDNA4/TO vector (Invitrogen, Waltham, MA, USA), respectively. The following primer sequences were used for the cloning of the cDNA : *Clock* sense primer 5´-TTTAAGCTTTCCTCACAGGAGGCGT GCGG-3´ and antisense primer 5´-TTTAAGCTT TCCTCACAGGAGGCGTGCGG-3´ ; *Bmal1* sense primer 5´-TTTGAATTCATGGCAGACCAGAGA ATGG-3´ and antisense primer 5´-TTTCTCGAG TTACAGCGGCCATGGCAAG-3´. The PCR conditions using Pyrobest polymerase (Takara Shuzo, Otsu, Shiga Prefecture, Japan) to amplify the cDNA of *Clock* were : 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C, followed by a final cycle at 72°C for 10 min. For amplification of *Bmal1* cDNA, the PCR cycling parameters were the same as above, except for the use of LA Taq polymerase (Takara Shuzo) pcDNA4/TO.*GFP.Bmal1* and pcDNA4/TO.*RFP.Clock* were generated by PCR amplification with primers containing in-frame BamHI and EcoRI site, and cloning into identically digested pcDNA4/TO.*Bmal1* and pcDNA 4/TO.*Clock*.

Establishment of Stable Bmal1/ Clock Transfected Cell Lines

pcDNA6/TR vectors (Invitrogen) were transfected into SW480 cells using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instructions. SW480-pcDNA6/TR cells were selected by 100 μM of Blasticidine (Invitrogen). pcDNA4/TO.*RFP.Clock* and pcDNA4/TO.*GFP.Bmal1* were transfected into SW480-pcDNA6/TR using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's protocols, and Zeocine (100 μM)-resistant clones were isolated using standard procedures. The pcDNA4/ TO.*RFP.Clock* and pcDNA4/ TO.*GFP.Bmal1* co-transfected cells were isolated and examined by fluorescent imaging using an OLYMPUS IX81 microscope with a GFP/Rhodamine filter set (IX2-RFAL and IX2-RFACA, OLYMPUS, Tokyo, Japan) after treatment of the cells with 30 μg/ml TET for 0-48 h. The stable transfected cell line was established after three rounds of subcloning. This cell line was defined as SW480/T-REx/*Clock*/*Bmal1*.

Production of Circadian Rhythm by Treatment with Dexamethasone

The method for induction of the circadian rhythm by treatment with dexamethasone was previously described[22]. SW480 cells were treated with 100 nM dexamethasone for 2 h, and then the medium was replaced with growth medium.

Conventional RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) from cells, and cDNA was synthesized with 2 μg of total RNA and random hexadeoxynucleotide primer (Invitrogen) in 20 μl of a solution containing reverse transcriptase. After synthesis, the cDNA was diluted 1 : 4 with distilled water and stored at −20°C until use. Each PCR was performed with cDNA derived from 20 ng of RNA. PCR reactions were carried out in a total volume of 20 μl containing cDNA, dGTP, dATP, dTTP, and dCTP at a concentration of 200 μM, 4 μM of each primer and 0.25 units of ExTaq polymerase (Takara Shuzo). The PCR primer used in this study is listed in Table 1. The PCR cycling parameters were as follows : 10 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C, followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel.

Quantitative Real-Time RT-PCR

For quantification, cDNA was used as a template in a real-time RT-PCR assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. *G3PDH* served as an internal control and was amplified parallel with every sample. The oligonucleotide primers for the detection of every gene expression and the *G3PDH* gene are listed in Table 1. The constituents of each PCR (15 μl) were 1 μl of cDNA, 1.0 μl of primer 15 pmol/liter each, 5.5 μl of distilled H_2O, and 7.5 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories).

Cell Count Assay

SW480 and SW480/T-REx/*Clock*/*Bmal1* cells were plated at a density of $3.0 \times 10^4/25\ cm^2$. When the cells were attached to the bottom of the flask, the medium was changed to growth medium with 30 mg/ml TET. After 48 h, the cells were harvested and counted every 24 h.

Flow Cytometry

Samples were prepared for flow cytometry. Briefly, cells were harvested, washed in ice-cold PBS (pH 7.4), fixed in ice-cold 70% ethanol, and treated with RNase (500 units/ml ; Invitrogen) at 37°C for 20 min. Cellular DNA was stained with 50 μg/ml of propidium iodide (Sigma Chemical Co., St. Louis, MO, USA), and cells were stored at 4°C. Cell cycle analyses were performed using a Becton Dickinson fluorescence-activated cell analyzer (Becton Dickinson, Mansfield, MA, USA). Cells (1×10^4) were analyzed for each point, and quantization of cell cycle distribution was performed using Modfit LT (Verity Software House, Topsham, ME, USA). All experiments were performed when cells reached 50% confluence.

Table 1.

Gene	Direction	sequence	product size (bp)	annieling temp.(℃)	MgClconc (mM)
Clock	F	agggtatttgccatttga	282	57	2
	R	gccaagttctcgtcgtc			
Bmal1	F	agacaatgaggggtgtaacc	338	57	2
	R	aaactgaaccatcgactccg			
Per1	F	gccagtgactttccaaca	333	57	2
	R	gaggcacatttacgcttagtg			
Per2	F	gatgccaaggtttgtggagttcctg	724	55	2
	R	gctctgtgagctcctgaatgctg			
Per3	F	gacagcagccacagtgaa	596	57	2
	R	acatgaatcagataggggggatg			
Cry1	F	atccgctgcgtcgtctacat	283	57	2
	R	atgacttctactccagcttca			
Cry2	F	gtgttcccaaggctggttctcc	324	57	2
	R	gtaggtctcgtcgtggttctcc			
CyclinD1	F	taagatgaaggagccatcccc	263	61	2
	R	ggattggaaatgaacttcacatctg			
G3PDH	F	cccctggccggtctcctgccttt	513	57	1.5
	R	ggcctgggtccccccccctgttgctgt			

Immunoblotting

Cells were lysed with ice-cold RIPA lysis buffer (150 ng NaCl, 5 mM Tris-HCl at pH 7.2, 1% Nonidet P-40, 1% sodium-deoxycorate, 0.05% Sodium dodecyl sulfate) and a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were adjusted by the cell number, 2.60×10^6 cells/1 ml RIPA buffer. Proteins were separated in 8% SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories). The transferred proteins were reacted with anti-RFP polyclonal antibody (Medical & Biological Laboratories CO, Nagoya, Japan), anti-GFP N-terminal developed in rabbit affinity isolated antibody (Sigma-Aldrich), and alpha-tublin (Sigma-Aldrich) at 4°C over night, followed by incubation for 30 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) or horseradish peroxidase-conjugated goat antimouse IgG (Vector Laboratories). Antibody binding was visualized with the ECL system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunoprecipitation

The cells were washed with ice-cold PBS, and the cytosol was lysed with buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 0.5 mM DTT) with proteinase inhibitor cocktail (Sigma-Aldrich). Deposits were lysed with buffer C (20 mM HEPES [pH 7.9], 20% (v/v) glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) with proteinase inhibitor cocktail (Sigma-Aldrich). The supernatants were diluted using distilled water and transferred to a fresh tube, and 2.5 μg of anti-GFP antibody (Sigma-Aldrich) or non-specific rabbit IgG was added, followed by incubation at 4°C for 1.5 h. Then, 30 μl of Protein G Sepharose (GE healthcare) was added and the mixture was incubated at 4°C for 1.5 h. The supernatants were discarded carefully, 1X sodium dodecyl sulfate sample buffer was added, and the samples were boiled for 5 min. They were then separated in 8% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories). Immunoblotting was performed with Anti-RFP antibody (Medical & Biological Laboratories CO) or anti-GFP antibody (Sigma-Aldrich) as a primary antibody, and horseradish peroxidase-conjugated anti-rabbit antibody as a secondary antibody. Antibody binding was visualized with the ECL system (GE Healthcare).

MTT Assays

The cytotoxicity of the anticancer drugs was measured using the MTT colorimetric assay (Sigma Chemical Co.). The cells were seeded in a 96-well plate with growth medium. On the following day, the medium was changed to 180 μl of RPMI 1640 containing 10% FBS and 30 μg/ml of TET. Thereafter, 20 μl of PTX at each concentration (30 nM-30 μM) was added to the wells (the final concentrations were 3 nM-3 μM) and incubations were continued at 37°C for four days, after which 50 μl of MTT (1 mg/ml PBS) was added to each well. After 4 h additional incubation at 37°C, the resulting formazan was dissolved in 90 μl of DMSO. Optical densities were read immediately at 570 nm by using a Micro Plate Reader (Bio-Rad Laboratories).

RESULTS

Induction of Circadian Rhythm in Human Colon Cancer Cell Line, SW480

We investigated whether SW480 cells show rhythm under dexamethasone treatment as described in the Materials and Methods section. The mRNA expression of circadian rhythm-related genes including *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* was examined by conventional RT-PCR (Fig. 1A). The mRNA levels of almost all genes were changed depending on time after treatment. We further performed real-time RT-PCR using the same samples (Fig. 1B). The mRNA levels of *Bmal1*, *Per1,* and *Cry1* were changed periodically, while *Clock* expression was not changed significantly. *Per1* mRNA expression seemed to be inversely correlated with *Bmal1* mRNA level.

Establishment of Both Clock and Bmal1 Stably Transfected Cell Lines

To observe the effects of *Clock* and *Bmal1* on the cell cycle, vectors including *Clock* or *Bmal1*, in which TET regulated the target gene expressions, were constructed as described in the Materials and Methods section. The expression of both *Clock* and *Bmal1* at the time after treatment with 30 μg/ml TET in the SW480/T-REx/*Clock*/*Bmal1* cells, was examined by conventional RT-PCR (Fig. 2A), real-time PCR (Fig. 2B), fluorescence microscopy and immunoblotting (Figs. 2C and 2D). The CLOCK/BMAL1 heterodimer activates transcription of *Per1* through binding to an E-box element in the *Per1* promoter region[9]; therefore, *Per1* expression was

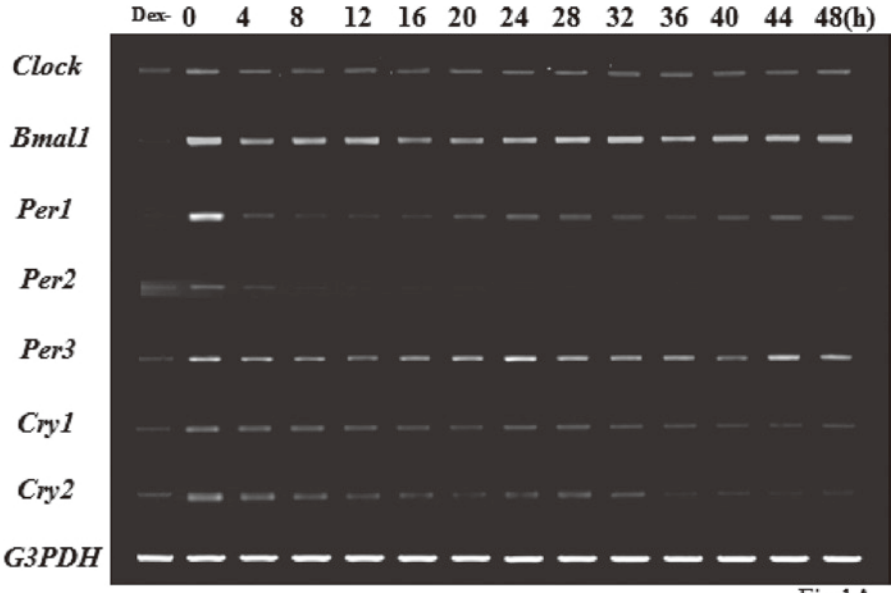


Fig.1A

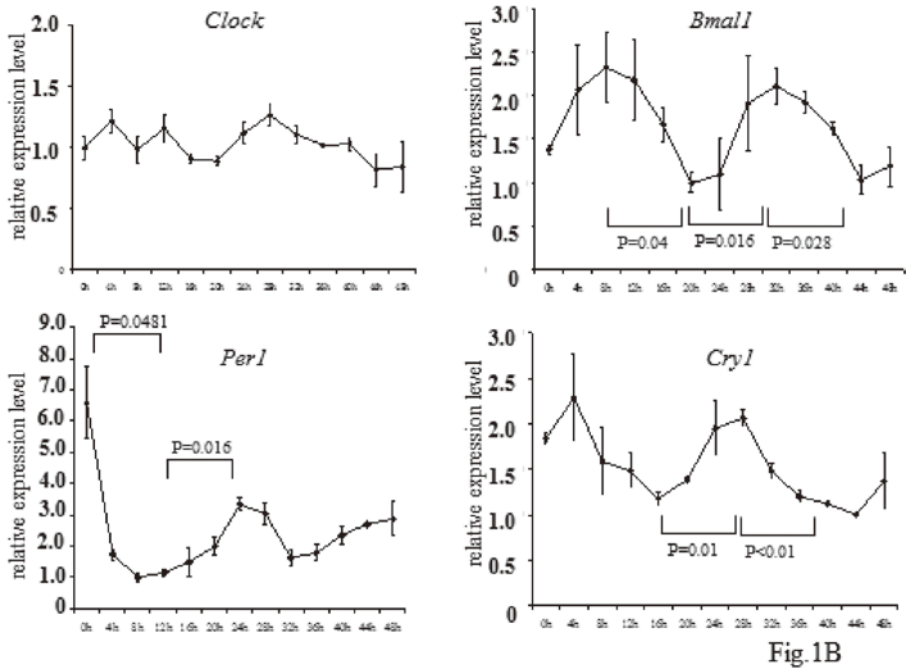


Fig.1B

Fig. 1. Circadian Rhythm in SW480 cells

Clock, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1,* and *Cry2* mRNA expressions of the SW480 cells after dexamethasone treatment were determined by conventional PCR (A) and real-time PCR (B). Each bar represents the mean ± SE (bars) of three independent experiments. Statistical differences were calculated by Student's *t*-test. *Bmal1*, *Per1*, *Per3*, *Cry1*, *Cry2* had rhythmical mRNA expression, whereas *Clock* did not (A, B). The expression of *Per2* was downregulated in this cell line (B).

used as a positive control in Figs. 2A and 2B. Both *Clock* and *Bmal1* expression levels were increased in a time-dependent manner with an increase of *Per1* after TET treatment in SW480/T-REx/*Clock*/*Bmal1* cells. Immunoprecipitation showed a CLOCK and BMAL1-formed heterodimer in our new cell line as already described[8,9] (Fig. 2E).

Overexpression of Both Clock and Bmal1 Suppressed Cell Growth

After establishment of the SW480/T-REx/*Clock*/*Bmal1* cells, we performed a cell count assay (Fig. 3). Cell growth was remarkably suppressed by both *Clock* and *Bmal1* induction. At 72 h, the growth ratio of the SW480/T-REx/*Clock*/*Bmal1* cells

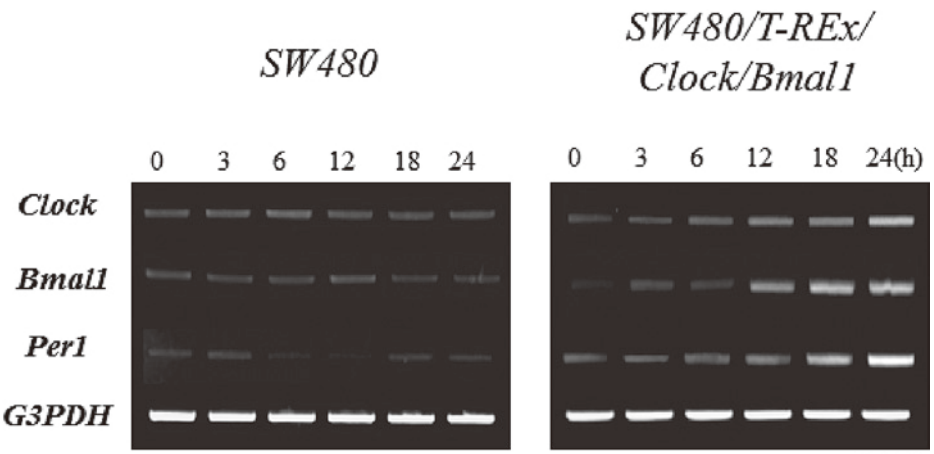
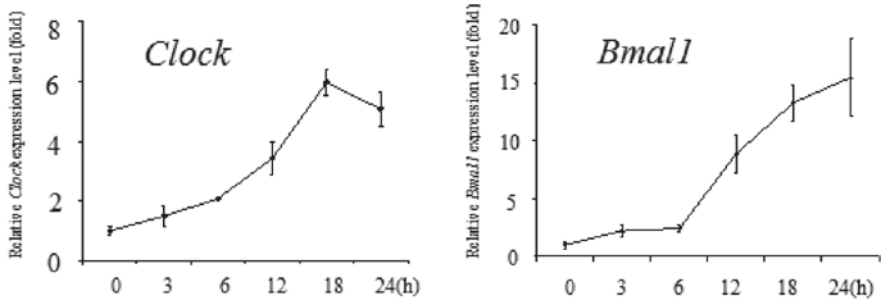


Fig.2A



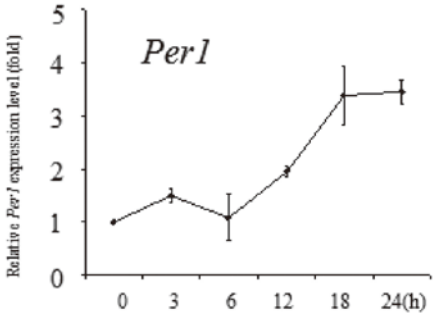


Fig.2B

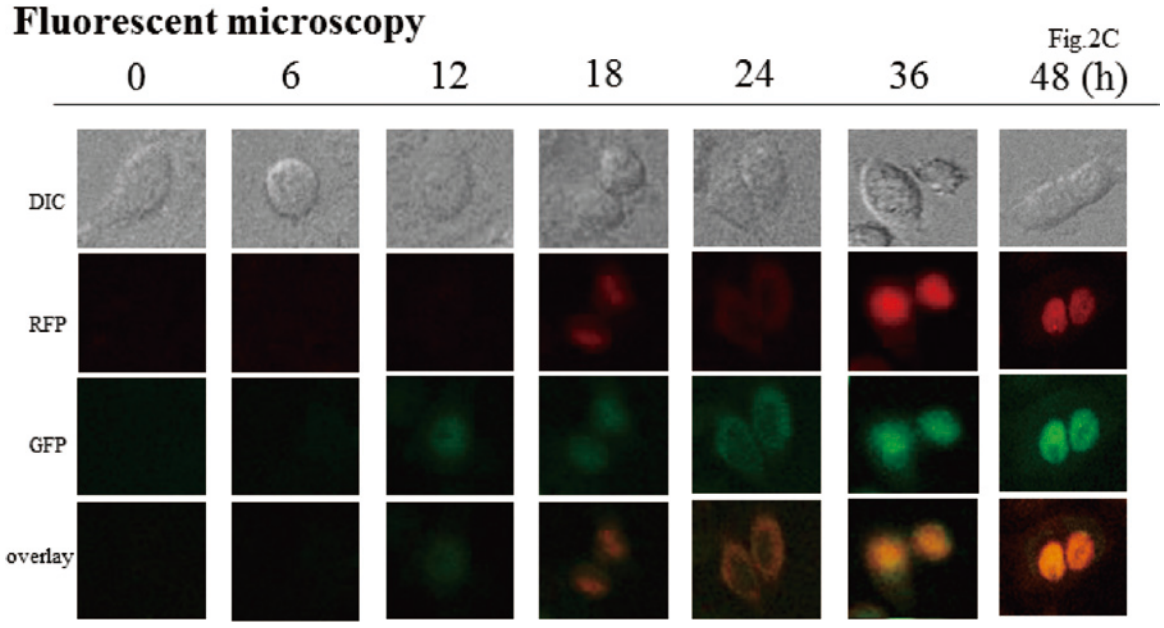


Fig.2C

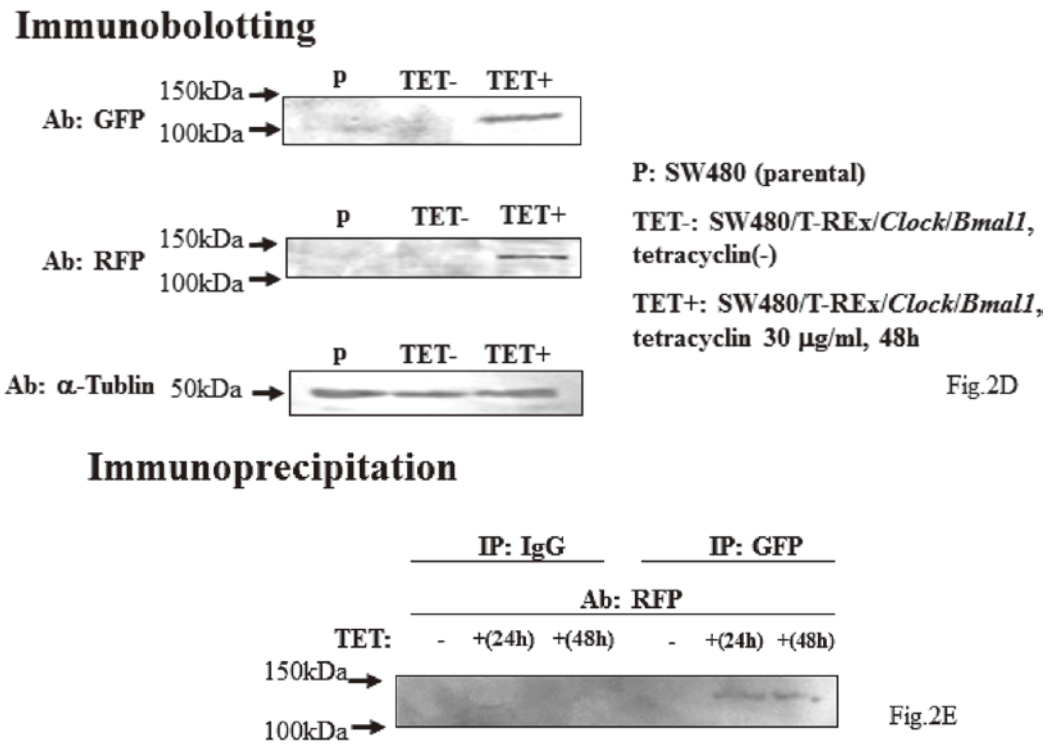


Fig. 2. Establishment of Stable Both *Clock* and *Bmal1* Transfected Cell Line
SW480 cells and SW480/T-REx/Clock/Bmal1 cells were plated at a density of 5.0×10^5/60 mm dish. After 24 h, the medium was changed to RPMI1640 supplemented 10% FBS and 30 μ the TET and harvested by TRIzol (Invitrogen) at 0, 3, 6, 12, 18, 24, 36, and 48 h after the medium change. cDNA was synthesized as described above. The expression of Bmal1 or Clock in the cells was observed by conventional RT-PCR (A), real-time PCR (B), fluorescence microscopy and immunoblotting (C). These data show that the mRNA of *Clock* and *Bmal1* was gradually induced, CLOCK and BMAL1 was produced gradually after TET treatment in the SW480/T-REx/*Clock*/*Bmal1* cells (D). Immunoprecipitation showed the CLOCK and BMAL1-formed heterodimer as already described (E).

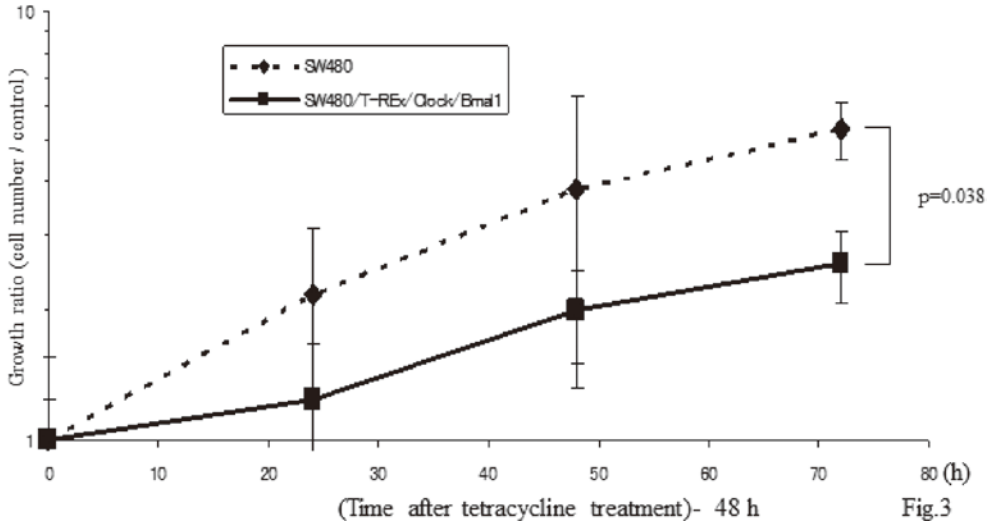


Fig. 3. Overexpression of Both *Clock* and *Bmal1* Suppressed Cell Growth
Each bar represents the mean ± SD (bars) of three independent experiments. Statistical differences were calculated by using Student's *t*-test.

was significantly less than that of the SW480 cells (5.32 ± 0.82 vs 2.57 ± 0.49, $p=0.038$).

Effect of Both Bamal1 and Clock Induction on Cell Cycle

To determine how the induction of these two

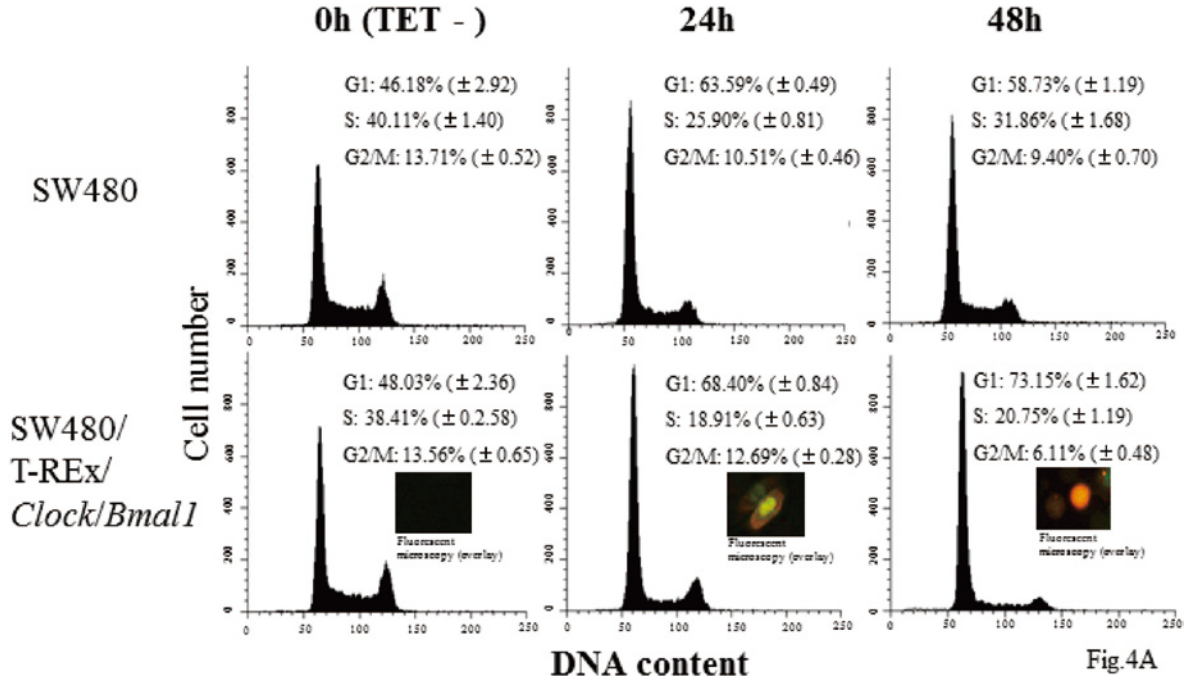


Fig.4A

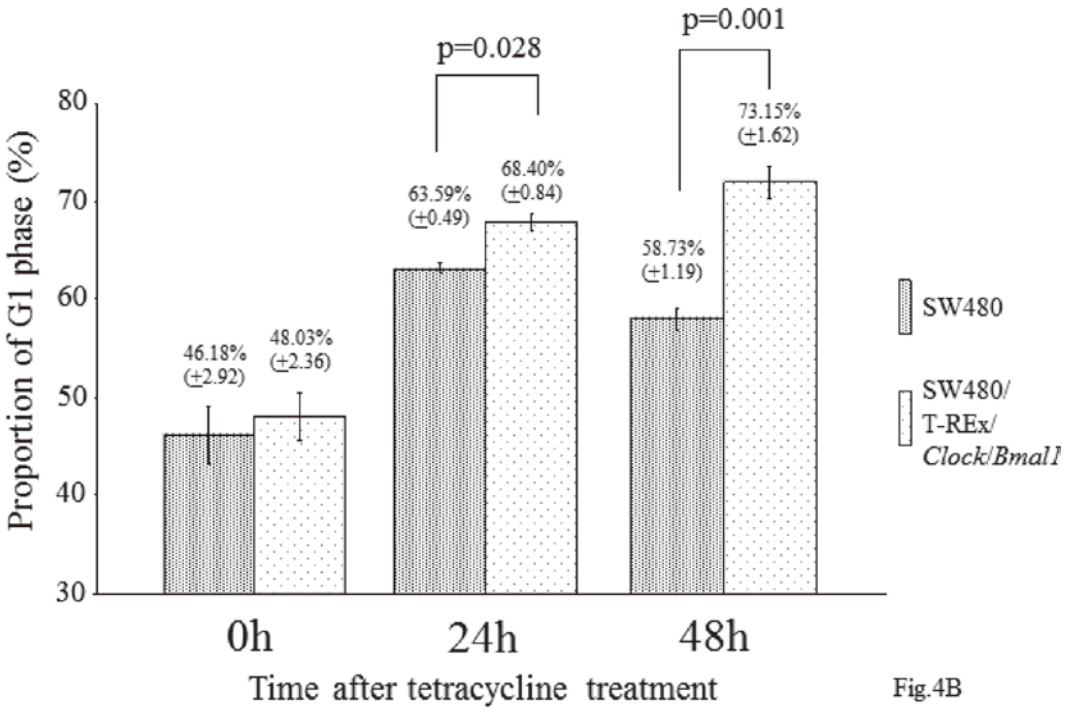


Fig.4B

Fig. 4. Effect of Both *Clock* and *Bmal1* Induction On Cell Cycle
SW480 cells and SW480/T-REx/*Clock*/*Bmal1* cells were plated at a density of $6.0 \times 10^5/75$ cm^2. After 48 h, the medium was changed to RPMI 1640 supplemented 30 μg/ml TET and 10% FBS. The samples were harvested and fixed for flow cytometry, as described above, every 24 h. A. Flow cytometric analysis showed a marked increased G1 phase cell proportion by the induction of *Clock* and *Bmal1* in the SW480/T-REx/*Clock*/*Bmal1* cells compared with the parental SW480 cells. Both cell lines were treated with 30 μg/ml TET for 0-48 h. B. The proportions of G1 phase in both cell lines were plotted. Each bar represents the mean ± SD (bars) of three independent experiments. Statistical differences were calculated by using Student's *t*-test.

genes suppresses cell growth, we first explored the effect of their induction on the cell cycle by flowcytometry. The proportions of the cells in G1, S and G2/M phase 24 h after TET treatment were 63.59 ± 0.49, 25.90 ±0.81 and 10.51 ± 0.46% in the SW480 cells, and 68.40 ± 0.84%, 18.91 ± 0.63 and 12.69 ± 0.28% in the SW480/T-REx/*Bmal1*/*Clock* cells. After 48 h, the G1, S, G2/M proportions were 58.73 ± 1.19, 31.86 ± 1.68% and 9.40 ± 0.70% in the SW480 cells, and 73.15 ± 1.62%, 20.75 ± 1.19 and 6.11 ± 0.48% in the SW480/T-REx/*Clock*/*Bmal1* cells (Fig. 4A). The proportion of G1 phase with and without TET treatment in both cell lines was plotted (Fig. 4B). The proportion of the cells in G1-phase in the SW480/T-REx/*Clock*/*Bmal1* cells (68.40 ± 0.84 % at 24 h, 73.15 ± 1.62% at 48 h) was significantly higher than that in the SW480 cells (63.59 ± 0.49% at 24 h, 58.73 ± 1.19% at 48 h) (p=0.028 at 24 h, p=0.001 at 48 h) (Fig. 4B). To demonstrate increased G1 proportion by *Clock* and *Bmal1* induction more clearly, we performed flow cytometry with cell cycle synchronization by serum starvation (Supplementary Fig. 1). These data indicated that overexpression of both *Clock* and *Bmal1* had an inhibitory effect on entry to the S phase.

Overexpression of Clock and Bmal1 Suppressed CyclinD1 Expression

To investigate how overexpression of *Clock* and *Bmal1* inhibits entry to the S phase, we examined *CyclinD1* expression level by real-time RT-PCR (Fig. 5). Before TET treatment, the *CyclinD1* expression level of the SW480/ T-REx/*Clock*/*Bmal1* cells was the same as that of the SW480 cells (1.0 ± 0.14 vs 1.06 ± 0.13, N.S.), but after TET treatment, the *CyclinD1* expression level of the SW480/T-REx/*Clock*/*Bmal1* cells was decreased in comparison with that of the SW480 cells in a time-dependent manner. At 48 h after TET treatment, the *CyclinD1* expression level of the SW480/T-REx/*Clock*/*Bmal1* cells was significantly decreased (0.70 ± 0.05 vs 0.38 ± 0.03, p=0.0048). These data suggested that both *Clock* and *Bmal1* overexpression might suppress *CyclinD1* expression directly or indirectly, and that entry to the S phase was inhibited due to the decrease of *CyclinD1* expression.

Overexpression of Both Clock and Bmal1 Change the Sensitivity to PTX

PTX, a clinically active anticancer agent isolated from the bark of the Pacific yew tree, inhibits mitotic spindle assembly or function, and the progression of mitotic cells to the G1 phase[18]. If overexpression of both Clock and Bmal1 has an inhibitory effect on entry to the S phase, the effect could compete for the pharmacological mechanism of PTX. We examined whether induction of these two genes altered the sensitivity to PTX. Without TET treat-

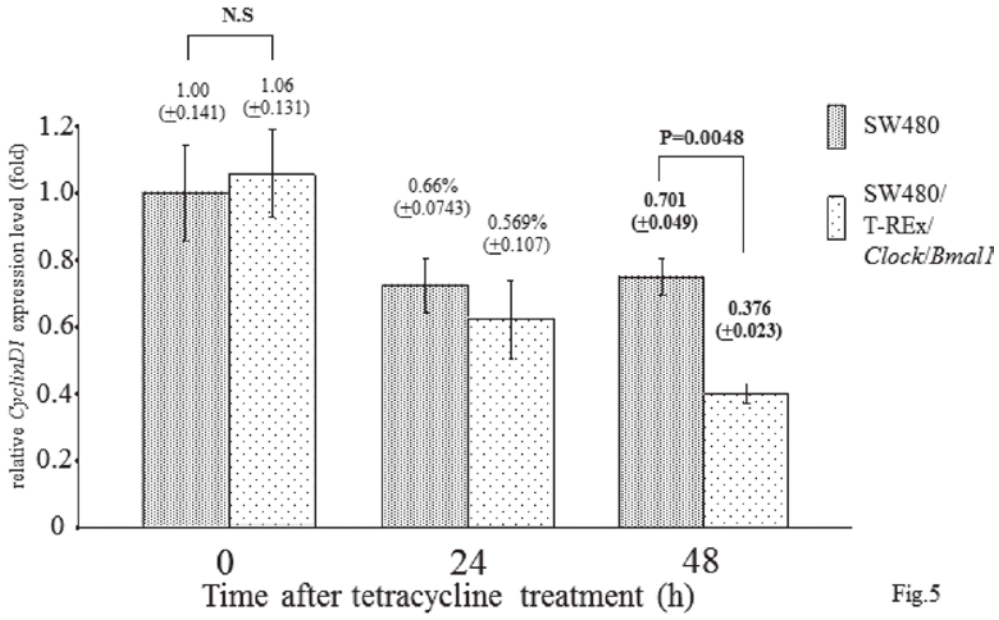
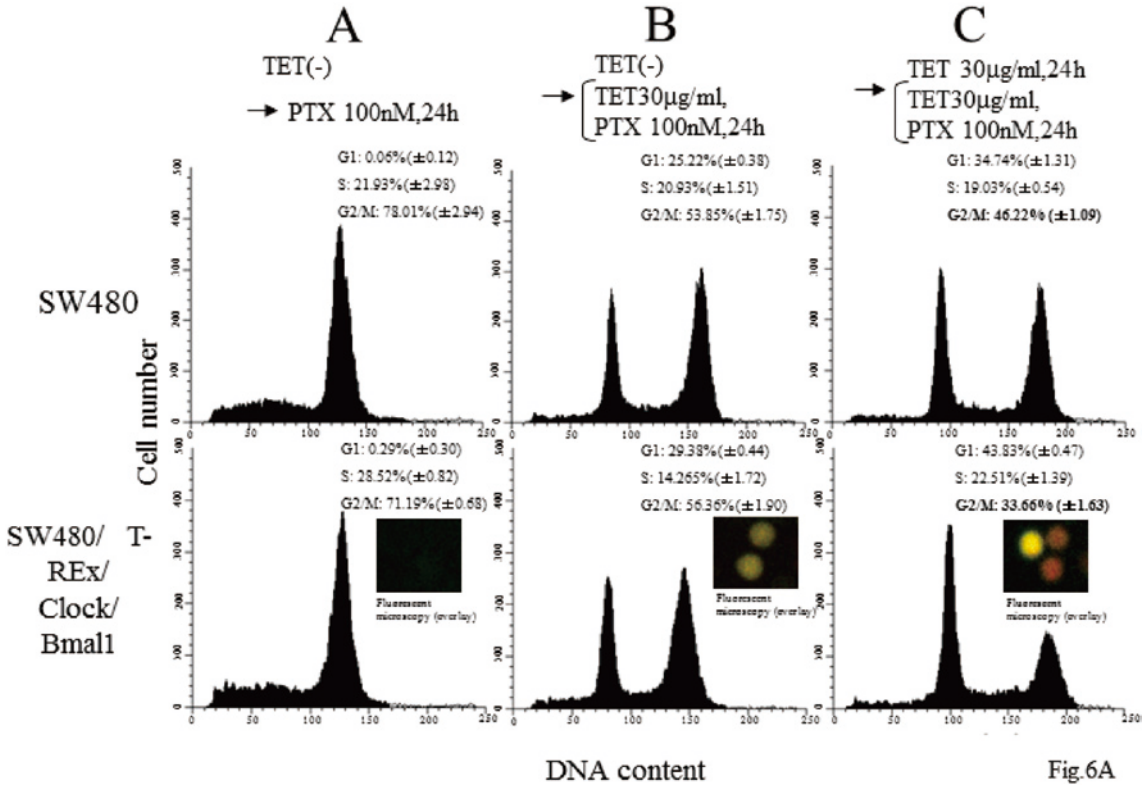


Fig. 5. *CyclinD1* Expression Level of *Clock* and *Bmal1* Overexpressed Cells
SW480 cells and SW480/T-REx/*Clock*/*Bmal1* cells were treated with 30 μg/ml TET for 0-48 h, and each *CyclinD1* expression level was observed by real-time PCR. *CyclinD1* was downregulated by *Clock* and *Bmal1* induction. Each bar represents the mean ± SD (bars) of three independent experiments. Statistical differences were calculated by using Student's *t*-test.



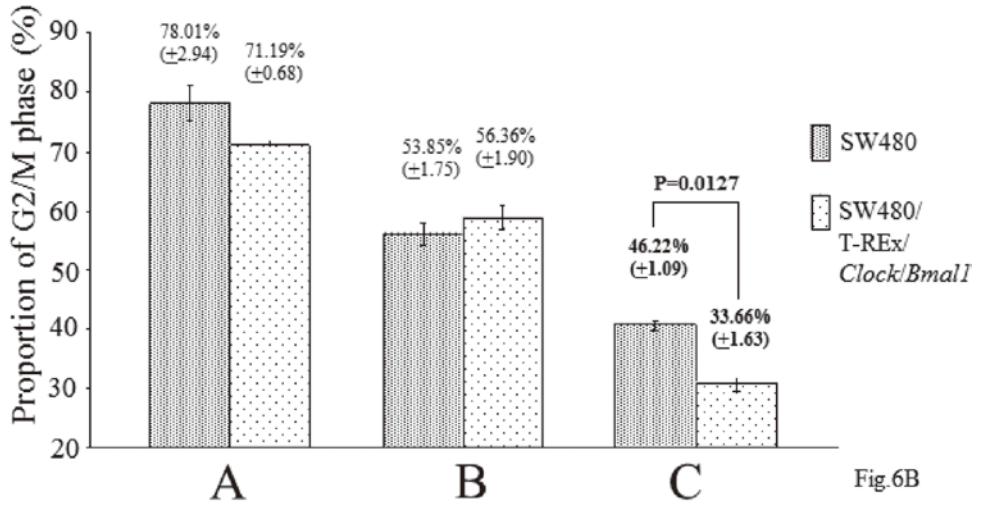


Fig. 6. Overexpression of *Clock* and *Bmal1* Prevents the Cells from Entry to G2/M Phase by Paclitaxel

A. SW480 cells and SW480/T-REx/*Clock*/*Bmal1* cells were plated at a density of 6.0×10^5/75 cm^2. (A) Cells were not pretreated with TET, but treated with PTX for 24 h, (B) Cells were not pretreated with TET but treated with 100 nM PTX and 30 μg/ml TET for 24 h, (C) cells were pretreated with 30 μg/ml TET for 24 h and treated with 100 nM PTX and 30 μg/ml TET for 48 h. The samples were harvested and fixed for flow cytometry described above. A. Flow cytometric analysis showed cell cycle arrest by 100 nM PTX in the SW480/T-REx/*Clock*/*Bmal1* cells and the parental SW480 cells. B. The proportion of the G2/M phase was plotted in a graph. Each bar represents the mean ±SD (bars) of three independent experiments. Statistical differences were calculated by using Student's *t*-test.

ment, PTX inhibited cell cycle progression in the G2/M phase of both the SW480 and SW480/T-REx/*Clock*/*Bmal1* cells equally. However, 24 h following TET treatment, the proportion of the G2/M phase in the SW480/T-REx/*Clock*/*Bmal1* cells was significantly lower than that in the SW480 cells (46.22 ± 1.09% vs 33.66 ± 1.63%, $p=0.0127$) (Figs. 6A and B). Furthermore, MTT assay showed that the induction of these two genes made the SW480 cells resistant to PTX (Fig. 7). Although TET itself has

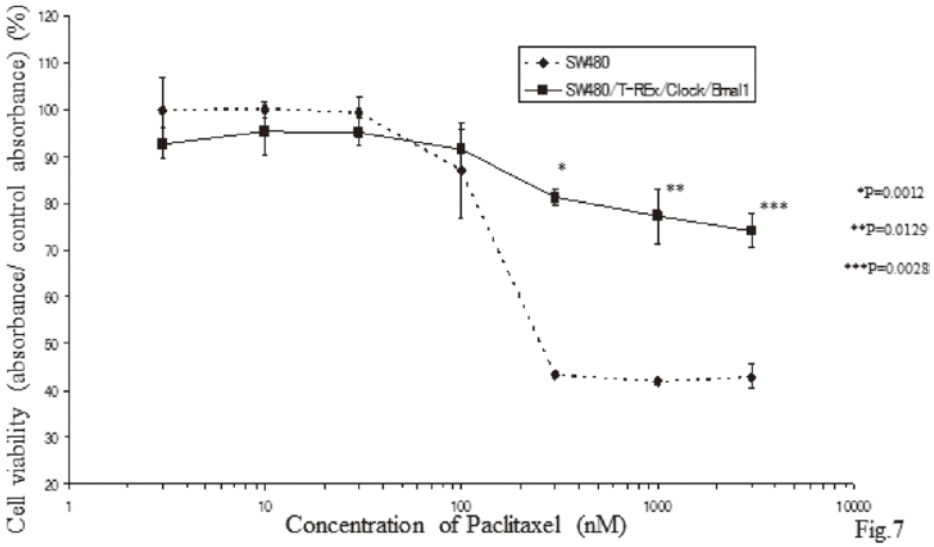


Fig. 7. Overexpression of both Clock and Bmal1 Makes SW480 More Resistant to Anticarcinoma Agent Paclitaxel
Each bar represents the mean ± SD (bars) of three independent experiments. Statistical differences were calculated by using Student's *t*-test.

an effect on the cell cycle to arrest at the G1 phase, the present data showed that simultaneous induction of *Clock* and *Bmal1* changes the chemosensitivity to PTX by inhibiting entry to the G2/M phase in SW480/T-REx/*Clock*/*Bmal1* cells.

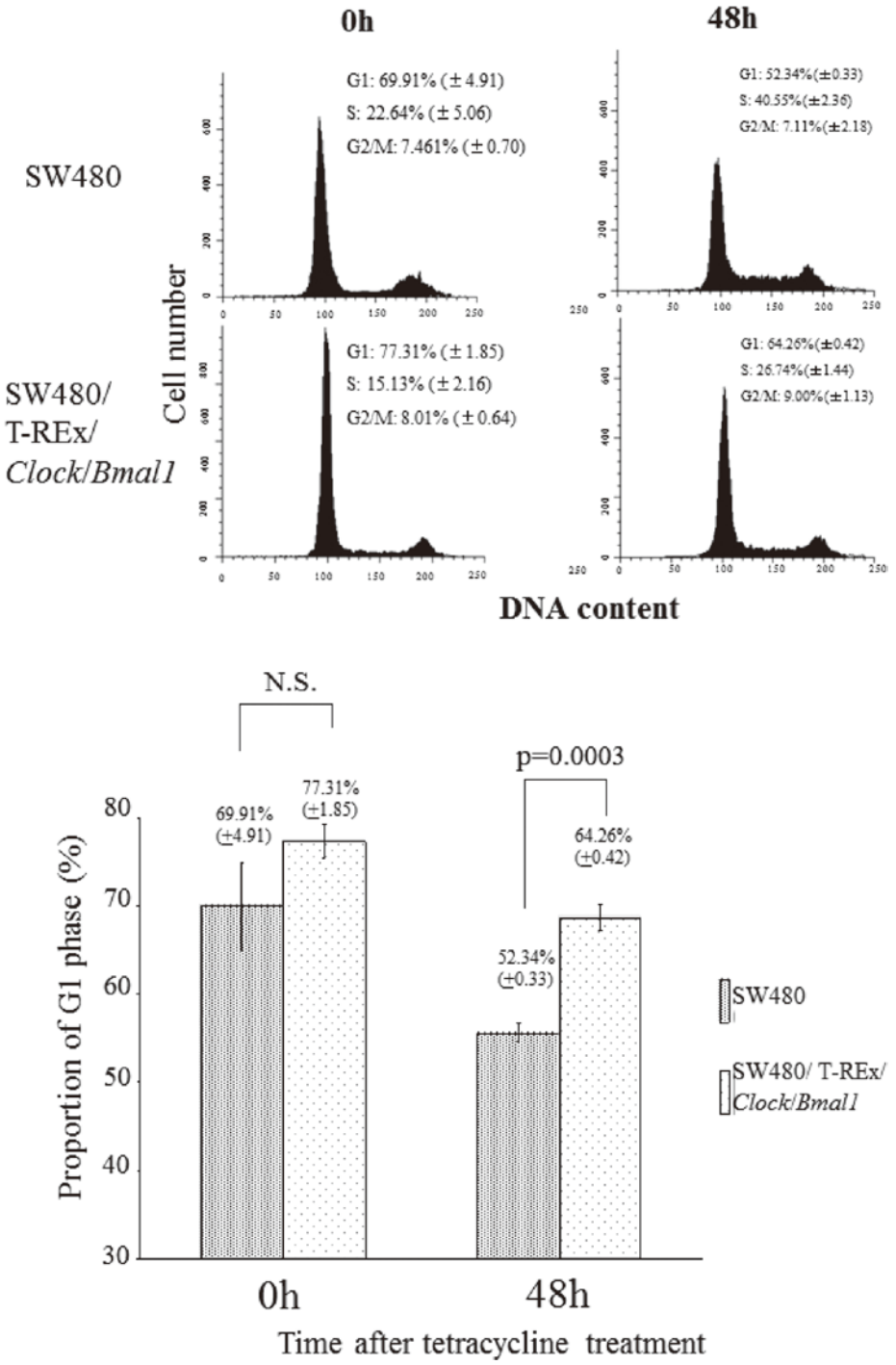
DISCUSSION

We confirmed that *Clock* and *Bmal1* form a heterodimer and promote *Per1* expression using SW480/T-REx/*Clock*/*Bmal1* cells. It is important to note "both *Clock* and *Bmal1*" not "either *Clock* or *Bmal1*", because the two proteins form a heterodimer, and bind to an E-box element at the promoter region of the target genes[9]. We showed that CLOCK and BMAL1 made a complex by immunoprecipitation. Kwon *et al.* reported that singly expressed CLOCK proteins were located in the cytoplasm but when CLOCK and BMAL1 were co-expressed they were found in the nucleus[23]; thus, Fig. 2C indicates that CLOCK and BMAL1 were dimerized in our cell line. One of the advantages of using the T-REx™ system is the gradual induction of the target genes in a time-dependent manner; we can observe the change that the genes cause more precisely by chasing the time course.

The major findings of the present study were as follows; i) overexpression of both *Clock* and *Bmal1* simultaneously inhibited cell growth, ii) the mechanism of slow cell growth was an inhibitory effect of these two genes on entry from the G1 phase to the S phase at cell cycle level, iii) in the *Clock* and *Bmal1* overexpressed cells, *CyclinD1* was downregulated, which may be a cause of increased G1 proportion, iv) overexpression of both *Clock* and *Bmal1* made cells more resistant to PTX.

We demonstrated that the simultaneous overexpression of both *Clock* and *Bmal1* inhibited cell growth possibly by inhibiting entry from the G1 phase to the S phase at the cell cycle level. To the best of our knowledge, this is the first report to indicate that *Clock* and *Bmal1* are involved in cell growth. Although Gorvacheva *et al.* have already produced *Clock* mutant/*Bmal*1 knockout mice, they did not report any growth abnormalities of the mice[16]. Matsuo *et al.* reported that liver regeneration of *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice is slower than that of wild type mice[20]. In comparison with these reports, our data is not necessarily contradictable, because in *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice, *Clock* and *Bmal1* expression levels should be relatively higher than those in wild type mice. In addition, it has been already reported that circadian clock genes *Per1* and *Per2* are involved in the G2/M or ATM checkpoints of the cell cycle[20,21], but there is yet to be report showing that the circadian gene is directly involved in the G1 checkpoint.

Gery *et al.* showed that PER1 interacted with Chk2, a key kinase of the ATM checkpoint pathway, and the expression levels of *Wee1* and *CyclinB1*, key molecules for the entry from the G2 phase to the M phase, were downregulated by overexpression of *Per1*[21]. Matsuo *et al.* reported that there were three E-box elements in the *Wee1* gene 5´-up stream



Supplemental figure. SW480 cells and SW480/T-REx/*Clock*/*Bmal1* cells were plated at a density of 1.0×10^6/75 cm^2. After 48 h, the medium was changed to RPMI 1640 medium supplemented 0.2% FBS and 30 μg/ml TET. Then, 48 h after the medium change, the medium changed to a growth medium-supplemented 30 μg/ml TET. The samples were harvested and fixed for flow cytometry every 24 h as described above. Each bar represents the mean ± SD (bars) of three independent experiments. The proportion of G1 phase was plotted in a graph. Statistical differences were calculated by using Student's *t*-test. These data strongly suggest that overexpression of both Clock and Bmal1 had an inhibitory effect on entry to the S phase.

region, and CLOCK and BMAl1 together produced a major increase in transcriptional activity of *Wee1*[20]. These reports were referred to G2/M checkpoint molecules. The G1 checkpoint is thought to be the most major checkpoint of the cell cycle, and *CyclinD* is the first response gene that connects growth signal and the start of the cell cycle. The expression of *CyclinD1* has reportedly been involved in the circadian system *in vitro* and in animal models[24-27] ; however, these reports simply showed that expression of *CyclinD1* had a circadian rhythm, and did not show that it detailed control mechanisms. Our data suggested that *CyclinD1* might be suppressed by the *Clock-Bmal1* heterodimer or by other genes activated by the one.

Of importance is the fact that the characteristics of the SW480/T-REx/*Clock/Bmal1* cells in the present study that should be mentioned are "gradual induction" and "not so much overexpressed *Clock* and *Bmal1*". In this cell line, *Clock* and *Bmal1* were gradually induced over a period of 24 h, and peak expression levels were about seven times (*Clock*) and 15 times *(Bmal1*) of the unsynchronized SW480 expression levels. Therefore, it was not so unnatural that *Clock* and *Bmal1* expression levels were about 10 times those of unsynchronized SW480, in comparison that Bmal1 expression level of synchronized SW480 changed between 1 to 2.5 times (Fig. 1B). Several reports suggest that mouse circadian rhythm, CLOCK and BMAL1 abundance are relatively higher at night[25,28,29]. The results of the present study may indicate possibility that the administration of PTX at night is not as effective as its daytime administration. To confirm this hypothesis, further studies are required.

In conclusion, our present data raised the possibility that *Clock* and *Bmal1* may have an effect on the G1 checkpoint of the cell cycle either directly or indirectly, and may change the sensitivity to PTX l of the human colon carcinoma cell SW480.

CONFLICT OF INTEREST DISCLOSURE

none.

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ABBREVIATIONS

Period1 (Per1), Period2 (Per2), Period3 (Per3), Cryptochrome1 (Cry1), Cryptochrome2 (Cry2), Bmal1 (ARNT-LILE PROTEIN 1, BRAIN AND MUSCLE), GFP (Green Fluorescent Protein), RFP (Red Fluorescent Protein)

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